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14. ABSTRACT
Culture-based and molecular-based studies highlight the diversity of bacterial pathogens in non-healing wounds, but these diverse bacterial communities have yet to be fully characterized. An enhanced understanding of the complex wound microbial communities is crucial to the development of next-generation wound diagnostics and therapeutics. The purpose of this project is to develop and apply cutting-edge molecular technologies to characterize wound microbiota in a non-biased, culture-independent fashion. To date we have: 1) Developed and applied a rapid, qPCR-based method for assessing bacterial load in wounds; 2) Developed and applied a novel, culture-independent pyrosequencing approach to characterize bacterial communities in wounds; 3) Pioneered an ecological-based statistical approach for analyzing microbial communities in a clinical context; 4) Revealed association between antibiotic therapy and increased *Pseudomonas* colonization in chronic wounds; and 5) Revealed association between diabetes and *Streptococcus* colonization in chronic wounds. We expect that our advances will facilitate a deeper understanding of the role of microbial colonization in wound healing and lead to more evidence-based wound therapies in the future.

15. SUBJECT TERMS
Antibiotics, bacteria, chronic wound, community analysis, diabetes, pyrosequencing, wound therapy, 16S rRNA gene

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Table of Contents

I. INTRODUCTION

Relatively minor wounds, received in both war and peace, can become infected with bacterial pathogens, leading to substantial morbidity and mortality. To date, both culture-based and molecular-based studies have highlighted the remarkable diversity of bacterial pathogens in non-healing wounds, but these diverse bacterial communities have yet to be fully characterized. An enhanced understanding of the complex wound microbial communities is crucial to the development of next-generation wound diagnostics and therapeutics. The purpose of this project is to develop and apply cutting-edge molecular technologies to characterize wound microbiota in a non-biased, culture-independent fashion.

II. BODY

II A. Develop qPCR for bacterial DNA

We have successfully developed a qPCR assay to accurately quantify bacterial DNA from mixed clinical samples. The 16S assay consisted of two primer pairs and a fluorescent probe. The selected primer and probe combination was, forward primer, 5'-TCCTACGGGAGGCAGCAGT-3' (Tm, 59 °C), the reverse primer, 5'-GGACTACCAGGGTATCTAATCCTGTT- 3' (Tm, 58 °C) and the probe, (6FAM)5'-TCAATCTGTCAATCCT-3'(MGB) (Tm, 69°C). This primer set generated an amplicon 467bp (340-807bp in the E. coli K-12 16S rRNA gene). qPCR was performed on the Applied Biosystems 7900 platform using optical grade 96 and 384-well plates. The PCR reaction was performed in a total volume of 25µL in 96-well plates and a 10µL volume in 384-well plates using Invitrogen qPCR Supermix with UDG. The primer and probe concentrations were 900nM of the forward and reverse primers and the 250nM of the fluorogenic probe. The reaction conditions for DNA amplification were 50°C for 2 min, 95°C for 4 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Data analysis made use of Sequence Detection Software version 2.3 supplied by Applied Biosystems.

Validation. For both the *E. coli* K-12 genomic and plasmid standards, the detection range was between 200fg and 2ng of genomic DNA, in terms of copies this was between approximately 34 and 340,000 copies. The distribution of replicates was within 0.2 Ct's of the median, this deviation only increased as a result of pipetting error from either poor tip sealing or using a non-calibrated pipette. The average number of cycles between replicates was at 3.35 with an R^2 value greater than 0.998, indicating 100% PCR efficiency of the standard. Data from running the two standards side by side was used to calculate the copy number/cell ratio. *E. coli* K-12 genome weight was calculated as 4.8fg/genome. Using this information we calculated 8 copies per genome, consistent with the published copy number of 7 in the rrnDB.

Sensitivity of the assay in detecting *Escherichia coli* rDNA. Taqman® assays allow determination of the PCR cycle at which the increase in fluorescence of the reporter dye reaches a threshold cycle (Ct). A Ct value is indicative of the log of the amount of target DNA, which is directly attributable to the log of the number of bacteria in the sample (adjusted to the number of gene copies per genome). The *Escherichia coli* standard had seven copies of rDNA in each copy of the chromosome, and had a mass of approximately 5fg. Using *Escherichia coli* as a standard, we consistently detected between 200 fg or 40 *Escherichia coli* cells and 2ng *Escherichia coli* DNA or approximately $4x10^5$ *Escherichia coli* cells. Although measurements outside of this range are possible, chances increase for a two-fold or more error in estimation. The greatest barrier to achieving greater assay sensitivity on the low end of the spectrum was contaminating bacterial DNA in commercially supplied reagents. The degree varied between different lots of the Invitrogen Supermix UDG, illustrating the importance of running several negative controls with each assay in order to determine the lower bound. The most likely explanation for this contamination is trace amount of DNA that makes it into reagents during polymerase preparation. We confirmed this contamination when observing rDNA in reagent mixes and negative controls containing no added bacterial DNA. To minimize this problem we attempted to use Environmental Master Mix from Applied Biosystems (which has been purified of all genomic DNA) however results from these runs yielded lower sensitivity that with regular master mix. We also attempted treating the Invitrogen Supermix with DNAse I (Invitrogen), then inactivating the enzyme by heating to 85°C for 15 minutes. Although this did remove late DNA amplification in the negative controls, it also degraded amplification efficiency in regular samples and had no improvement in assay sensitivity.

Detection and quantification of panel DNAs. We determined that the universal assay did not amplify human DNA (obtained from Applied Biosystems), plant DNA (from a Pinyon Pine tree), or fungal DNA (representatives of four fungal divisions were tested). This broad panel also allowed us to determine that variations in rDNA copy number had no direct correlation to differences copy between standardized samples when using this assay. When tested against the 21 panel organisms, the assay reliably amplified all members of the panel. Once standardized, 17 of the 21 members of the panel amplified within 1.5-2 Ct's of the standard, resulting in a quantity difference of 40%. *Borrelia burgdorferii* and *Coxiella burnetii* amplified an average of four cycles later than the *E. coli* standard, indicating an underestimation of genomic quantity by 100-150%. *Mycobacterium pneumoniae* and *Anabaena variabilis* consistently amplified 9 Ct's from the standard, underestimating the actual genomic quantity by 1000-fold. PCR efficiency was initially checked for each DNA by multiplexing the 16S assay with the Exogenous IPC from Applied Biosystems. Doing this decreased sensitivity and only worked well with Applied Biosystems Taqman® Master Mix. To overcome this problem we opted to run the IPC independently and verify results by creating five serial ten-fold dilutions of each isolate and checking for 100% PCR efficiency along these dilutions.

All isolates exhibited no inhibition with the IPC assay. In addition all isolates except for *Borrelia burgdorferii*, *Coxiella burnetii*, *Mycobacterium pneumoniae,* and *Anabaena variabilis* had 100% +/- 10% PCR efficiency among serial dilutions. Although no inhibition was detected when using the IPC, *C. burnetii* and *B. burgdorfeii* amplified with approximately 3.5, 3.7, and 4 cycle differences between serial dilutions from 2ng to 2pg of genomic DNA, and had an average efficiency of only 85%. *Mycobacterium pneumoniae,* and *Anabaena variabilis* similarly had no detectable inhibition when checked with the IPC, however performing a serial dilution down from 2ng of DNA resulted in >4 Ct difference, both isolates amplified the 200pg quantity at the trace DNA threshold of cycle 34.

We also tested for amplification anomalies as a result of complex mixtures composed of multiple organisms, we ran groups of DNAs in a mixture of 2, 4, 6, and 8 standardized genomic DNA extracts from different bacteria. These runs yielded results that were directly proportional to the sum of the concentrations of individual DNAs (data not shown). However, when mixtures of larger numbers of DNAs were attempted, the total DNA concentration exceeded 1ng/µL and the intervals between serial dilutions of these mixtures decreased. The logarithmic relationship was restored by diluting individual DNAs and re-pooling into groups of 10 and 12 organisms. More complex mixtures were not attempted because at extremely low dilutions there was no qPCR-independent method of verifying individual DNA concentrations added to the total mixture.

The *E. coli* genomic DNA standard was mixed with 1%, 10%, 50%, 90%, and 99% *Saccharomyces cereviciae* DNA then with again with the same ratios of human DNA. There was slight inhibition of the standard at the 99% non-target concentration of both human and *S. cerevisiae* DNAs. This experiment was repeated with the mixtures of 4 panel DNAs and yielded the same results. Following these experiments the assay was tested with an additional panel of 30 pathogens that commonly cause Community Acquired Pneumonia/SEPSIS. All organisms in that group amplified within 1.5 – 2 Ct's of the standard and were accurately quantified based on earlier measurements of genomic DNA quantity.

II B. Calculate Human:Bactial DNA ratio

Bacterial load is considered a critical aspect of wound healing. Traditionally, bacterial load is calculated by weighing, homogenizing and culturing wound biopsy tissues. Load is then reported per mg of tissue. This method is not conducive to calculating bacterial load based on swab samples and relies on the accuracy of balances that are typically not calibrated to weigh tissue samples on the mg scale. In order to develop a more accurate and repeatable method for measuring bacterial load from both tissue and swab samples, we developed a method whereby bacterial load can be estimated based on the ratio of bacterial DNA to human DNA. Bacterial DNA is measured using our custom, in-house qPCR assay for the 16S gene (described in quarter 1 report) and human DNA is measured using a qPCR assay for the human *Alu* gene. The 16S gene copy number can be used to determine the approximate number of bacteria in a sample. Likewise, the *Alu* gene copy number can be used to estimate the number of human cells in a sample. Finally, bacterial load is calculated by dividing the number of bacterial cells to the number of human cells.

Bacterial to Human Cell Ratio: calculation methods. The wound samples were extracted using the protocol "Isolation of Bacterial Genomic DNA from Clinical or Environmental Samples" (version 1.4). Once extracted, the samples were amplified using ABI Internal Positive Control (IPC) kit to detect the presence of PCR inhibitors. No inhibitors were detected. The bacterial load was quantified by real time-PCR using 16S target specific primers and prope (developed in quarters 1 and 2) against dilutions (neat to 1:100 dilution). A plasmid standard curve with 10-fold dilution was added to each plate (10^8 to 10^2 copies of 16S gene). The number of copies of the 16S gene was calculated based on the standard curve and the neat values were back-calculated including the respective dilution factor. The number of bacterial genomes was calculated by dividing the copy number by 4.13 (the average number of 16S genes in an individual bacterium).

The human cell load was quantified using the *Alu* Human Target qPCR Assay. This assay targets the human *Alu* subfamily Ya-5 gene using the primers Forward: GACCATCCCGGCTAAAACG, Reverse: CGGGTTCACGCCATTCTC and probe 6FAM-CCCCGTCTCTACTAAA-MGBNFQ on the LightCycler® 480 Real Time PCR Instrument (Roche). The samples were tested with dilutions (neat to 1:100 dilution) and were run along side a plasmid standard curve of 10-fold dilutions (10^8 to 10^2 copies of the *Alu* gene). To calculate the human cell load for each sample the quantity from the qPCR results was multiplied by the dilution factor to generate the calculated or expected copy number in a neat sample, this calculated neat copy number was then divided by 5000 (the number of copies of the *Alu* subfamily Ya-5 gene found in a diploid human genome or cell).

The ratio of bacterial to human cells was also calculated for each sample by dividing the number of bacterial genomes by the number of human genomes.

Results. We used the qPCR method described above to calculate the bacterial load in 32 chronic wound samples. Bacterial loads ranged from 0.03 to 5184 (average, 191) bacteria per human cells. Fourteen of the wound samples were from patients who had received antibiotics within the past two weeks. We stratified samples by recent exposure to antibiotics and compared bacterial load using a Mood's median test (The data were not normally distributed; therefore, s Mood's median test was run on the data). There was a significant difference in the median bacterial load for patients who took antibiotics versus patients who did not take antibiotics (*p*-value = 0.033) (figure 1).

Conclusions. The qPCR-based bacterial load assay that we have developed is a rapid, culture-independent method for calculating bacterial load in wounds. This method can be used for a wide array of clinical samples including swab, curette and biopsy samples from acute and chronic wounds. Using the ratio of bacterial to human DNA frees the clinical researcher from having to estimate tissue weights or swabbed surface area for bacterial load calculations. Here we have applied it to chronic wound samples and detected a significant reduction in bacterial load associated with recent antibiotic exposure.

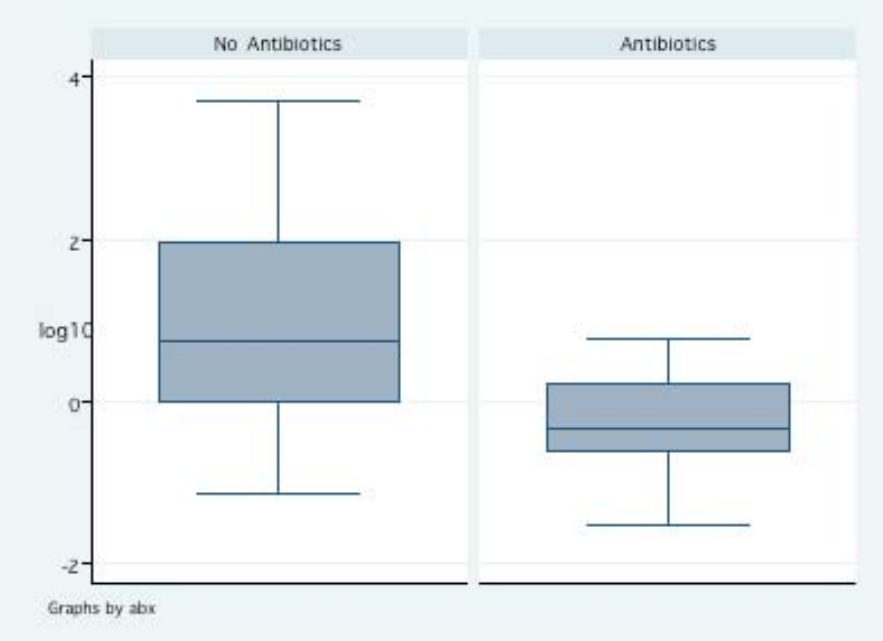

Figure 1. Box plot of $\log_{10}$ transformed bacterial load data from patients with and without recent exposure to antibiotics.

II C. Develop Pyrosequence Data Analysis Pipeline

Recent advances in pyrosequencing technology provide the potential to characterize complex bacterial communities in a culture-independent fashion at depths not possible previously. One of the greatest challenges to utilizing this platform is having the bioinformatic and statistical capabilities necessary to make use of the hundreds of thousands of DNA sequences that are created in each run. Over the past year, we have developed a data analysis pipeline for handling 16S rRNA gene sequences from the Roche FLX454 pyrosequencer. The methods described below will be published in a manuscript later this month (appendix B).

Experimental sequences were processed using a custom PERL script, which performs the following: the script filtered the sequence files and retained only sequences that were 200-nt or longer. It then searched for a single barcode sequence in each FASTA sequence, binned each sequence accordingly, and scanned each binned sequence for the 16S forward primer sequence. The script then trimmed off the forward primer sequence and oriented the remaining sequence such that all sequences begin with the 5' end according to standard sense strand conventions. As a result of our processing, sequences that were shorter than 200-nt or had multiple barcode or primer motifs were excluded from the analysis. We included only sequences with the forward primer motif to ensure that the highly informative V3 region was available for taxonomic assignment. The trimmed sequences from each barcode bin were aligned using the NAST alignment tool (http://greengenes.lbl.gov) [DeSantis 2006]. After alignment, the number of sequences examined per wound

sample was equilibrized to 300 sequences by sampling randomly without replacement to facilitate subsequent taxa abundance analyses. Samples with fewer than 300 sequences were excluded. The cutoff of n = 300 was established based on richness (rarefaction) and diversity (Shannon-Weaver Index) analyses using DOTUR [Schloss 2005], which indicated that samples were sufficiently sampled after ≥300 sequences.

Taxonomic assignment. Unaligned, sequences in the equilibrated dataset were given taxonomic assignments at a bootstrap confidence range of ≥95% using the Ribosomal Database Project's Naïve Bayesian Classifier tool (RDP classifier) [Wang 2007; Cole 2009].

Rarefaction and diversity analyses. Distance matrices based on taxa abundance were generated with the Dnadist tool of PHYLIP 3.67 using the default settings [Felsenstein 1989]. Rarefaction and Shannon Weaver index estimations were determined by DOTUR [Schloss 2005] and plotted in Microsoft Excel (Microsoft Corp., USA).

Statistical analyses. All statistical analyses were performed using our equilibrated dataset (n = 300 sequences per sample). Community-scale multivariate analyses including non-metric multidimensional scaling (nMDS), multiresponse permutation procedure (MRPP), and the Dufrêne & Legendre indicator analyses were performed in R [R Development Core Team 2008] using statistical packages vegan [Oksanen 2009], ecodist [Goslee 2007], BiodiversityR [Kindt 2005], and labdsv [Roberts 2007]. The nMDS analysis is a nonparametric ordination-based method for reducing ecological community data complexity and identifying meaningful relationships amongst communities, while the MRPP analysis is another nonparametric method for testing the null hypothesis of no-difference between communities by comparing the experimental with the expected within-group difference through an iterative randomization process. The indicator species analysis further identifies the bacterial taxa that are significantly unique to each environment (e.g., clinical variables of interest). The nonparametric nature of these ecological analysis methods is highly suitable for human bacterial community data, which are frequently zero-rich, highly-skewed, and non-normal and remains non-normally distributed post-data transformation. Significance level for MRPP and the Dufrêne & Legendre indicator analyses were set at $\alpha = 0.05$.

Comparative analysis of mean indicator prevlaence between environments (e.g. diabetics versus non-diabetics) were also performed in R using custom codes. Briefly, using the taxa abundance-based distance matrices, a t-statistic was calculated and the underlying null distribution was estimated using Monte-Carlo based resampling (n = 10,000 permutations). A two-tailed empirical p-value was generated by comparing the unpermuted data with the estimated null distribution. Significance levels were set at $\alpha = 0.05$ with the appropriate Bonferroni correction (α/n), with n = number of tests performed for a single environment.

Assessment for the interaction between antibiotic use and diabetes and the percent agreements in the comparison of 16S rRNA gene-based and culture-based results were performed using multivariate logistic regression and the kappa-statistic, respectively in STATA 9 (StataCorp, USA).

II D. Pyrosequence bacterial communities from wound samples.

In addition to developing the pyrosequencing analysis pipeline described above, we have also developed a highly efficient method for analyzing complex bacterial communities using the 16S rRNA gene sequence. The methods described below will be published in a manuscript later this month (appendix B).

Pyrosequencing library synthesis for parallel tagged sequencing on the 454® platform. The 16S rRNA gene was amplified in two replicate 50 µl reaction volumes. In each 50 µl reaction, 3 µl was added to 47 µl of PCR reaction mix containing 450 nM of each broad range forward (5'-CCTACGGGAGGCAGCAGT-3') and reverse primer (5'-GGACTACCAGGGTATCTAATCCTGTT-3') [Nadkarni 2002], 1X PCR buffer without $MgCl_2$ (Invitrogen), 3 mM $MgCl_2$, 0.2 mM dNTP mix, 0.02 U platinum *Taq* (Invitrogen) using the following touch-down PCR condition: 90s at 95°C for initial denaturation, 30s at 95°C for denaturation, 30s at 64°C for annealing, 30s at 72°C for extension with the annealing temperature decreasing by 0.3°C for each subsequent cycle for 34 cycles, followed by 5 min at 72°C for final extension. Subsequent purification, blunt-end repair, adapter ligation, amplicon quantification and pooling, restriction digestion, and pyrosequencing library generation were carried according to a previously published protocol [Meyer 2008]. The sample-specific, palindromic, self-hybridizing barcodes used in the tagging reactions were generated using a self-complementary 8-nt barcode and a rare restriction site according the same protocol.

Pyrosequencing using the 454® platform. The pooled tagged single-stranded pyrosequencing library underwent fusion PCR and pyrosequencing using a Roche 454 FLX Pyrosequencer (Roche Life Sciences, USA) according to the manufacturer instructions [McKenna 2008] at the Institute for Genome Sciences, Genomic Resource Center.

II E. Clone 16S & Clone Sequencing

Prior to the pyrosequencing method (described above), clone libraries were considered the gold standard for culture-independent, molecular characterization of bacterial communities. We used this method to describe bacterial communities from chronic wounds as described below. We found that both pyrosequencing and clone library analysis produced similar results, but pyrosequencing could provide much greater depth of analysis at a fraction of the cost.

16S rDNA-based clone library construction of wound samples. PCR primers were used to amplify the 16S rDNA from genomic DNA extracted from chronic wound samples provided by Johns Hopkins Wound Center. For clone library construction, primers AllBact_F1 (5′-CICCTACGGGIGGCWGCAG-3′, positions 338 to 357 of *E. coli*) and AllBact_R1 (5′-GGACTACCIGGGTATCTAATCCYITT-3′, positions 781 to 806 of *E. coli*) were used. All PCR thermocycling was carried out using Platinum *Taq* High-fidelity polymerase (Invitrogen Corp., Carlsbad, CA) in a DNAengine thermocycler (BioRad Laboratories, Hercules, CA). Each 50 µL PCR reaction contained: about 30 ng of purified genomic DNA, 200 µmol of each dNTP, 5.0 µL of 10x PCR Buffer, 3 mM Mg^{2+}, 450 nM of each primer, 1.0 U polymerase and 35.55 µL sterile 18 MΩ water. Amplification of 16S rDNA using the AllBact_F1/R1 primer set was achieved with a touch-down thermocycling program that consisted of a 1.5 min denaturation cycle at 95˚C followed by 33 cycles of 30 seconds at 95˚C, 30 seconds of annealing from 64˚C to 52˚C, stepping down 0.4˚C each cycle and 60 seconds at 72˚C, with a final elongation cycle of 72˚C for 5 minutes. PCR products were purified using the AMPure SPRI-based PCR purification kit according to the manufacturer's recommendations (Agencourt Bioscience, Beverly, MA). Concentrations of purified products were determined by PicoGreen assay (Invitrogen) measuring fluorescence using an ND3300 Fluorometer (NanoDrop, Willmington, DE). Cloning and transformation of competent E. coli cells using PCR products from the AllBact_F1/R1 reactions was performed with the TOPO TA Cloning Kit for Sequencing (Invitrogen) according to the manufacturer's recommendations. The pCR 4-TOPO® vector was used in conjunction with TOP-10 chemically competent cells of *E. coli* (Invitrogen). After transformation, 200 µL of new S.O.C. media (Invitrogen) and the cells were incubated at 37˚C for one hour. Following incubation, 100 µL from each transformation was spread onto LB agar plates supplemented with 50 µg mL^{-1} kanamycin (Teknova, Hollister, CA). Plates were incubated overnight at 37˚C. Clone colonies were picked from the agar using sterile pipette tips. Picked clones were archived into individual wells of a 96-well microtiter plate containing 200 µL LB broth with a 7.5% concentration of glycerol (Teknova) and supplemented with 50 µg mL^{-1} kanamycin. After overnight incubation at 37°C with shaking, the archive plates were stored at -20°C

Plasmid preparation of clone libraries. Archived cell libraries were used to inoculate 384 well culture plates (Fisher Scientific) containing 200 µL of LB broth supplemented with 50 µg mL^{-1} kanamycin (Teknova). Each block was covered with breathable sealing tape and incubated for ~24 hours at 37°C with shaking at 400 rpm. Cells were pelleted by centrifugation at 4000 rpm for 10 min using an Eppendorf 5810R fitted with rotor model A-4-62. The supernatant was then removed by inversion of the blocks. 25 µL of GTE solution with RNAse A (100 µg mL^{-1}) was added and the cells were resuspended by vortexing. Fifty microliters of lysis solution 1 (0.2N NaOH, 1% SDS, made fresh from concentrated stock solutions) was added to each well. Wells were mixed by gentle shaking, then incubated at room temperature for 5 minutes. After incubation, 25 µL of 3M potassium acetate solution was added to each well followed by gentle shaking, then incubation for 5 minutes in a -20°C freezer. After freezer incubation, the plates were centrifuged for 20 minutes at 4,000 rpm. The supernatant from each well (75 µL) was transferred to a new 384 well microplate (Fisher) already holding 50 µL of cold isopropanol per well. The plates were incubated for 20 minutes in a -20°C freezer. Following incubation, the plates were centrifuged for 10 minutes at 4000 rpm. The supernatant was then decanted off and 75 uL of 70% ethanol was added to each well. The plates were centrifuged (10 minutes, 4000 rpm), the supernatant was poured off and the pellets were washed again. Following the second ethanol wash, the plates were allowed to air dry before the purified plasmid DNA was resuspended in 20 µL of filter sterilized 1X TE (pH 8.0, Fisher Scientific, Pittsburg, PA). Resuspended plasmid preparations were stored at -20°C until used for downstream applications.

Sanger sequencing and phylogenetic analysis of clone inserts. The 16S rDNA insert of fourty-eight plasmid preps from each wound library was amplified from the T7 promoter (5'-TAATACGACTCACTATAGGG-3') and T3 primer (5'-ATTAACCCTCACTAAAGGGA-3') sites on the TOPO-TA vector using recombinant *Taq* polymerase (Invitrogen) following the manufacturer's recommended concentrations and conditions. PCR products were stored at -20°C until further use. The following functions were performed by the TGen Sequencing Center (TGSC, Scottsdale, AZ). The T3/T7 PCR products were purified using solid phase

reversible immobilization (SPRI)-based technology (AMPure®; Agencourt Biosciences Corp., Beverly, MA), resulting in the removal of unincorporated dNTPs, primers, and salts. PCR products were eluted in 30μl distilled H_2O. Both strands of each PCR product were sequenced as follows: Sequencing reactions were performed using 3μl (approximately 25ng) of purified PCR product in a 6μl reaction containing 0.33μl BigDye Terminator v3.1 premix, 3.2 pmol of T7 primer, and 1.03μl 5X BigDye sequencing buffer. Cycle-sequencing was performed for 35 cycles following the manufacturers recommendations on GeneAmp 9700 PCR machines (Applied Biosystems, Foster City, CA). Sequencing reactions were purified using CleanSEQ® (Agencourt Biosciences Corp., Beverly, MA) to remove unincorporated dye-terminators, and analyzed on 3730xl DNA analyzers (Applied Biosystems, Foster City, CA). Sequences from each wound library were analyzed using a PERL script we designed (RST v.1.4). The script searched each .seq file received from the TGSC for the AllBact_F1 and R1 sequence motifs and trimmed off any vector sequence as well as orienting all fragments so that the AllBact_F1 sequence is found at the 5′ end of each trimmed sequence. The script also filtered out any sequences that had ambiguous primer motifs, poor sequence quality and reads shorter than 400nt in length. Trimmed sequences from each library were aligned using the NAST alignment tool (DeSantis 2006. NAST) found on the GreenGenes database website (http://greengenes.lbl.gov). Each sequence was compared against their database with at most one non-redundant uncharacterized near neighbor reference and two non-redundant near neighbor references from isolate species retrieved for each sequence submitted. Query sequences were submitted in groups of 100 to 500 to minimize the number of redundant reference returns over the whole library. NAST aligned sequences were imported into the phylogenetic database program ARB (Ludwig 2004), running on the Osiris server, which is a 64-Bit SMP machine with 4 dual-core AMD processors and 24 GB of memory running Redhat Enterprise and located at the Translational Genomics Research Institue High Performance Biocomputing Center (TGen HPBC, Phoenix, AZ). ARB was used to construct phylogenetic trees representing the 16S rDNA associations made by clones of each wound library to reference sequences retrieved from GreenGenes. This was achieved by first constructing a tree using only the reference sequences. The clone sequences were then imported and parsimoniously added to the reference tree by filtering out all but the region of the reference sequences where the cloned 16S fragments had aligned. Graphical and text enhancements of ARB trees were performed using Xfig v.3.2.4.

Rarefaction and abundance analysis. Sequences from each library were aligned as described above and used as the input for the Dnadist tool of PHYLIP 3.67 [Felsenstein, J. 1989] in order to generate distance matrices for each library. Rarefaction and Chao I richness estimations were determined by DOTUR [Schloss 2005] using the distance matrices from each library as input. The rarefaction and Chao I results were plotted using Microsoft Excel (Microsoft Corp., Redmond, WA). PHYLIP and DOTUR analysis was performed on the Osiris server mentioned above.

UniFrac analysis. Wound libraries were examined for β diversity associations using the program UniFrac (Lozupone 2005). These analyses were performed on a phylogenetic tree representing the associations between all of the clones without the presence of the nearest neighbor reference sequences. Clone operational taxanomic units (OTUs) were given environmental assignments according to the wound of origin. Using these environmental assignments, the tree was interrogated for meaningful associations between clone libraries. The 'clusterEnvironments' analysis was used to generate UPGMA clustering results. A tree representing the unweighted comparison of the different wounds was generated. This analysis takes into account the presence and absence of species between environments and groups the environments according to similarity of community structure. The resulting Newick formatted trees were visualized using NJplot and annotated using The Gimp 2.4. The UniFrac PCA analysis was used to generate principle component results from a comparison of the community differences between wounds. The unweighted analysis was performed using the default parameters. The principle components that explained the greatest amount of variation between environments were plotted.

Richness Estimations. In order to estimate sequence diversity in each sample, we generated rarefaction curves from our 16S rRNA gene clone libraries using three different cutoff levels for operational taxonomic units (OTUs): unique genus level (Figure 2). Rarefaction estimates indicated that we did not sample a sufficient number of clones to fully describe diversity at the unique sequence and species levels, but curves appear to be stabilizing for most samples at the genus level. Even as such, the diversity estimates generated using the 16S clone library sequences indicate a much greater level of microbial diversity than was measured by culture. The added depth provided by the Roche 454 platform will permit us to sample wound communities to a sufficient depth as to fully describe diversity at the species level.

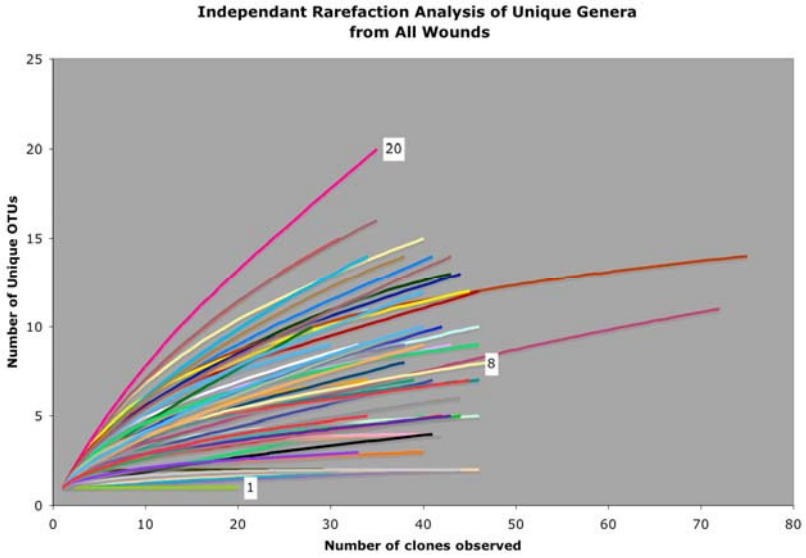

Figure 2. Rarefaction Analysis of Unique Genera from 16S clone libraries.

Wound Type Analysis. In order to test the hypothesis that different wound types have distinctive microflora, we performed three different analyses on our 16S rRNA gene clone library data. First, we used principle coordinate analysis to determine if wound type (neuropathic foot ulcer, venous stasis ulcer, decubitus ulcer, and other) could explain a significant portion of the community variation observed between the samples (Figure 3). This analysis revealed no significant association between wound types. Next, we divided observed species into oxygen tolerance groups (aerobic, anaerobic and facultative anaerobes; microaerophilic organisms were not observed) and then used the non-parametric Wilcoxon Rank-Sum (Mann-Whitney) test to compare ratios of oxygen tolerance groups between wound types in a pair-wise manner (Table 1). Ratios of oxygen tolerance types were not significantly different between the three wound types when compared to one another. These findings were supported using one-way ANOVA (data not shown). Finally, we compared the rate of colonization with group B Streptococcus between the different wound types using Chi^2 test to compare rate ratios, but again there were no statistically significant associations.

Diagnosis	Proportion of Oxygen Types Mean (SD)	vs NFU (*p* value)	vs VSU (*p* value)
Anaerobes			
NFU	0.159 (0.201)		
VSU	0.050 (0.098)	0.293	
DEC	0.312 (0.405)	0.473	0.160
Aerobes			
NFU	0.214 (0.293)		
VSU	0.516 (0.432)	0.119	
DEC	0.251 (0.171)	0.310	0.327
Facultative Anaerobes			
NFU	0.628 (0.266)		
VSU	0.437 (0.371)	0.206	
DEC	0.434 (0.385)	0.317	0.806

Table 1. Proportion Oxygen Tolerance Types, Pair-wise Comparison by Wilcoxon rank-sum test. NFU, neuropathic foot ulcer; VSU, venous stasis ulcer; DEC, decubitus ulcer.

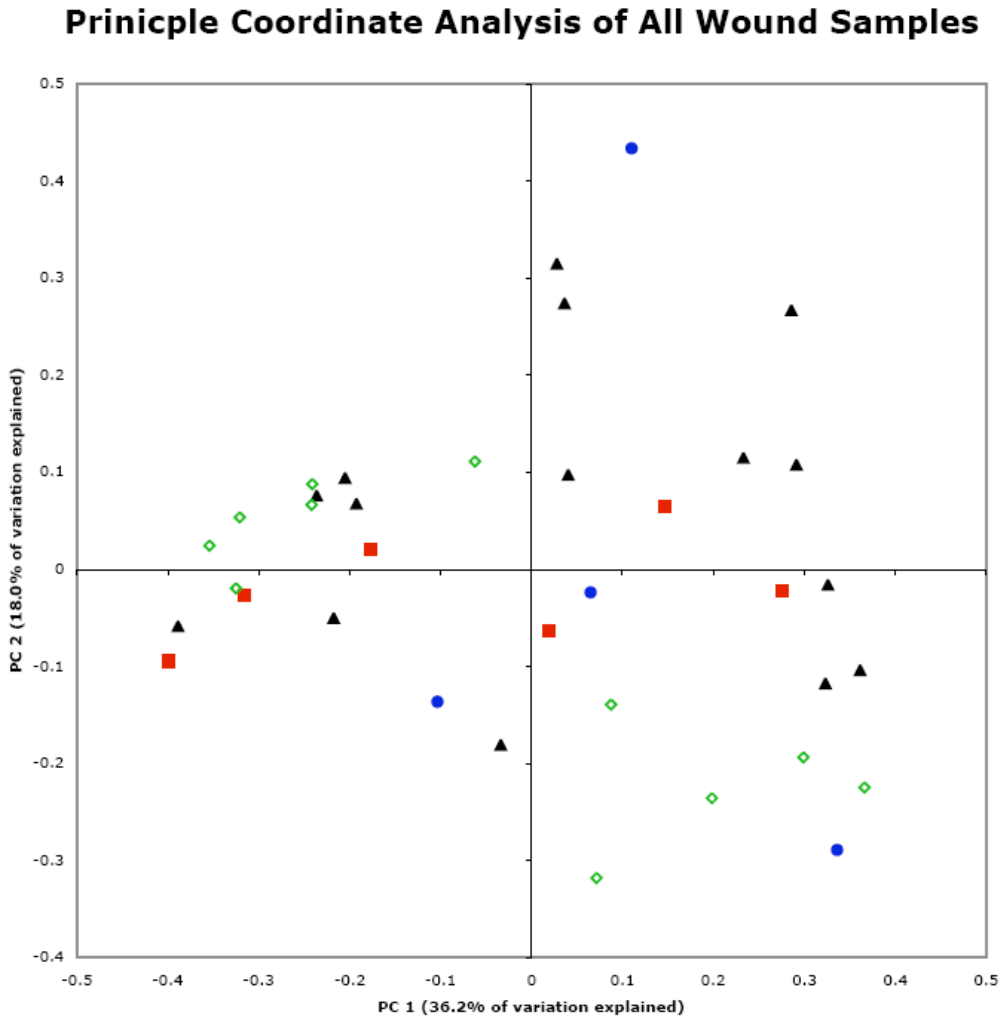

Figure 3. Principal Coordinate Analysis of Wound Type. Solid black triangles, neuropathic foot ulcer; Closed red squares, venous stasis ulcer; Closed blue circles, decubitus ulcer; Open green diamonds, other wound types.

Characteristic	Group B Strep*		Pair-wise Comparisons	
	Yes	No	NFU	VSU
NFU (n=15)	5	10		
VSU (n=5)	1	4	1.67 (0.251, 11.1); p = 0.573	
DEC (n=4)	0	4	Infinity; p = 0.179	0.0; p = 0.343

Table 2. Group B Streptococcus Positive Wound Types. NFU, neuropathic foot ulcer; VSU, venous stasis ulcer; DEC, decubitus ulcer.

II F. Analyze samples with the PhyloChip

The microarray-based PhyloChip is another culture-independent, molecular tool for characterizing complex microbial communities. We are in the process of testing this method on wound microflora and have just finished running our samples on the PhyloChip (note: phylotype assignments have not been completed). PhyloChip analysis was conducted as described previously (Flanagan 2007):

Amplification of the bacterial 16S rRNA gene. The 16S rRNA gene was amplified from extracted DNA with the universal bacterial primers Bact-27F (5-AGAGTTTGATCCTGGCTCAG -3) and Bact-1492R (5- GGTTACCTTGTTACGACTT -3) (Lane, 1991). The reaction mixture (50 μl, final volume) contained 5 μl of 10□ PCR buffer, 1 □μl of deoxynucleoside triphosphates (10 mM), 0.7 μl of forward primer and reverse primer (100 pmol/μl each), 0.35 μl of Taq poμlymerase (5 U/μl), and 1 μl of template DNA. PCR was performed with the DNA Engine thermal cycler (Bio-Rad). To maximize the number of bacterial species that could be recovered by PCR, three different annealing temperatures (48°C, 52°C, and 56°C) were used for each sample to amplify the 16S rRNA genes. The following cycling parameters were used: 3 min of initial denaturation at 95°C followed by 25 cycles of denaturation (30 s at 95°C), annealing (30 s), and elongation (120 s at 72°C), with a final extension at 72°C for 7 min. Amplified products from all samples were verified by gel electrophoresis. All PCR products were gel purified with the QIAquick gel extraction kit (QIAGEN), and, for each sample, the purified products amplified at three different annealing temperatures were pooled for cloning, sequencing, and microarray analysis.

PhyloChip processing, scanning, probe set scoring, and normalization. PhyloChip processing, scanning, probe set scoring, and normalization. The pooled PCR product was spiked with known concentrations of synthetic 16S rRNA gene fragments and non-16S rRNA gene fragments as internal standards for normalization, with quantities ranging from 5.02×10^8 to 7.29×10^{10} molecules applied to the final hybridization mix. Target fragmentation, biotin labeling, PhyloChip hybridization, scanning, and staining were as described by Brodie et al. (Brodie 2006), while background subtraction, noise calculation, and detection and quantification criteria were essentially as previously reported (Brodie 2006), with some minor exceptions. For a probe pair to be considered positive, the difference in intensity between the perfect match (PM) and mismatch (MM) probes must be at least 130 times the squared noise value (N). A taxon was considered present in the sample when 90% or more of its assigned probe pairs for its corresponding probe set were positive (positive fraction, ≥0.90). Hybridization intensity (referred to as intensity) was calculated in arbitrary units for each probe set as the trimmed average (maximum and minimum values removed before averaging) of the PM minus MM intensity differences across the probe pairs in a given probe set. All intensities of <1 were shifted to 1 to avoid errors in subsequent logarithmic transformations. To account for scanning intensity variations from array to array, the intensities resulting from the internal standard probe sets were natural log transformed. Adjustment factors for each PhyloChip were calculated by fitting a linear model by the least-squares method. A PhyloChip's adjustment factor was subtracted from each probe set's lnintensity.

III. KEY RESEARCH ACCOMPLISHMENTS

- Developed and applied a rapid, qPCR-based method for assessing bacterial load in wounds
- Developed and applied a novel, culture-independent pyrosequencing approach to characterize bacterial communities in wounds
- Pioneered an ecological-based statistical approach for analyzing microbial communities in a clinical context
- Revealed association between antibiotic therapy and increased *Pseudomonas* colonization in chronic wounds
- Revealed association between diabetes and *Streptococcus* colonization in chronic wounds

IV. REPORTABLE OUTCOMES

- Presented results at the 48th Annual ICAAC/IDSA 46th Annual Meeting (see Appendix A)
- Prepared a manuscript that has been accepted for publication in PLoS One (See Appendix B)

V. CONCLUSION

The work that we are conducting is aimed at moving wound research beyond the limitations of culture-based microbial analyses to enable comprehensive characterization of wound colonization and to fully evaluate its impact on healing. We have succeeded in developing a novel, culture independent approach for characterizing wound microbiota. We have also succeeded in applying statistical tools—originally developed for the complex data analysis challenges of ecology (e.g., nMDS)—to begin characterizing the impact of antibiotic therapies and diabetes on wound microbiota. We expect that our advances will facilitate a deeper understanding of the role of microbial colonization in wound healing and lead to more evidence-based wound therapies in the future.

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VII. APPENDICES

APPENDIX A. ABSTRACT

Price LB[1,2], Melendez JH[1], Frankel YM[1], Wang NY[1], Bowers J[2], Ravel J[3], Keim P[2] Lazarus GS[1], Zenilman JM[1].
16s-Based Molecular Characterization of Chronic Wound Microflora to Improve Future Therapies. The 48th Annual ICAAC/IDSA 46th Annual Meeting scheduled on October 25-28, 2008 in Washington, DC

BACKGROUND Chronic wounds are wounds that do not progress through the normal healing process and exist for months to years. In the US, treatment of chronic wounds is estimated to cost between 8 and 15 billion dollars annually. While host factors and co-morbidities are important predictors of chronic wounds, bacterial colonization is also considered a critical factor in the chronic non-healing state. 16S gene-based methods may provide greater resolution for wound microflora characterization compared to standard culture-based methods.

METHODS We enrolled 28 chronic wound patients at the Johns Hopkins Wound Center. Curette samples were collected at enrolment and ~2 weeks after standard medical therapy. Standard cultures were prepared from each wound sample in CLIA certified laboratories. V3 and V4 regions of the 16S gene were amplified from curette samples by PCR. PCR products were used to generate 16S clone libraries and pyrosequencing libraries. ~48 clones from each wound were analyzed by Sanger sequencing. 1000 to 4000 pyrosequencing reads were generated from each wound sample.

RESULTS 16S-based methods regularly revealed 5-10 fold more complex bacterial communities in chronic wounds as compared to culture-based methods. Wound microflora varied substantially between patients with some wounds colonized predominantly by aerobes while others were colonized largely by anaerobes. Bacterial complexity varied from 2 to >30 sequence types within a wound.

CONCLUSIONS Organisms revealed by 16S analysis, but undetected by culture may play critical roles in the non-healing state of chronic wounds. A more complete understanding of wound microflora may translate to better, more evidence-based wound therapies in the future.

APPENDIX B. MANUSCRIPT
Accepted for publication in PLoS One

TITLE
Community Analysis of Chronic Wound Bacteria using 16S rRNA Gene-based Pyrosequencing: Impact of Diabetes and Antibiotics on Chronic Wound Microbiota

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PLoS one

Community Analysis of Chronic Wound Bacteria Using 16S rRNA Gene-Based Pyrosequencing: Impact of Diabetes and Antibiotics on Chronic Wound Microbiota

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Abstract

Background: Bacterial colonization is hypothesized to play a pathogenic role in the non-healing state of chronic wounds. We characterized wound bacteria from a cohort of chronic wound patients using a 16S rRNA gene-based pyrosequencing approach and assessed the impact of diabetes and antibiotics on chronic wound microbiota.

Methodology/Principal Findings: We prospectively enrolled 24 patients at a referral wound center in Baltimore, MD; sampled patients' wounds by curette; cultured samples under aerobic and anaerobic conditions; and pyrosequenced the 16S rRNA V3 hypervariable region. The 16S rRNA gene-based analyses revealed an average of 10 different bacterial families in wounds—approximately 4 times more than estimated by culture-based analyses. Fastidious anaerobic bacteria belonging to the Clostridiales family XI were among the most prevalent bacteria identified exclusively by 16S rRNA gene-based analyses. Community-scale analyses showed that wound microbiota from antibiotic treated patients were significantly different from untreated patients ($p = 0.007$) and were characterized by increased Pseudomonadaceae abundance. These analyses also revealed that antibiotic use was associated with decreased Streptococcaceae among diabetics and that Streptococcaceae was more abundant among diabetics as compared to non-diabetics.

Conclusions/Significance: The 16S rRNA gene-based analyses revealed complex bacterial communities including anaerobic bacteria that may play causative roles in the non-healing state of some chronic wounds. Our data suggest that antimicrobial therapy alters community structure—reducing some bacteria while selecting for others.

Citation: Price LB, Liu CM, Melendez JH, Frankel YM, Engelthaler D, et al. (2009) Community Analysis of Chronic Wound Bacteria Using 16S rRNA Gene-Based Pyrosequencing: Impact of Diabetes and Antibiotics on Chronic Wound Microbiota. PLoS ONE 4(7): e6462. doi:10.1371/journal.pone.0006462

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Chronic wounds cause substantial morbidity and economic burden that is borne disproportionately by diabetic, geriatric, and immobilized patients [1]. Generally associated with venous, arterial, or metabolic abnormalities [2], more than 90% of the chronic wounds fall into three categories: diabetic ulcers, venous ulcers, and pressure ulcers [3]. Four processes have been hypothesized to be the underlying cause in chronic wounds: 1) local tissue hypoxia, 2) repetitive ischemia-reperfusion injury, 3) altered cellular and systemic stress response, and 4) bacterial colonization [3]. Among these, bacterial colonization is of particular interest to clinicians for its association with chronic wound infections and as targets for novel wound therapies.

Although bacterial colonization occurs in all chronic wounds, the differentiation between *wound colonization* and *invasive infection* is not well defined. The hypothesized impact of bacterial colonization on wound healing ranges from detrimental to beneficial depending on the colonizing bacterial species and relative load [4]. Wound colonization is typically polymicrobial in nature (i.e., consisting of multiple bacterial species) [5]; thus, broad-spectrum antibiotics may modify, but not eliminate bacterial colonization.

Clinicians routinely use antibiotics for chronic wound care, but their optimal use and benefit remain unclear [6]. Systematic reviews have found little evidence for the benefit of antibiotic therapy on wound healing [7,8]. Yet, patients with chronic wounds continue to receive more antibiotic therapy than age- and sex-matched non-wound patients [9], even as antibiotic-resistant organisms such as methicillin-resistant *Staphylococcus aureus* and antibiotic-resistant *Pseudomonas* are becoming more prevalent in wounds [10]. Assessment of the impact of antibiotic use in chronic

wounds will be crucial to establishing an effective, evidence-based regimen and to minimize inappropriate antibiotic use.

Traditionally, wound microbiota has been defined using culture-based methods; however, these methods are insufficient for characterizing complex polymicrobial communities, since many microbes cannot be cultured. There is an increasing number of chronic conditions and diseases associated with non-culturable or fastidious bacteria including bacterial vaginosis [11]; Whipple's disease [12]; and reactive arthritis [13]. Thus, it is critical to better define the role of fastidious and non-culturable bacteria in chronic wounds. Advanced molecular-based techniques, such as 16S rRNA gene-based pyrosequencing, can be used to characterize complex bacterial communities independent of culture-based enrichment. Preliminary 16S rRNA gene-based surveys of bacterial species associated with chronic wounds have found many putative wound colonizers that were not detected using standard culture-based methods and have revealed previously undescribed levels of bacterial diversity in chronic wounds [14–16]. Yet, these studies were mostly descriptive due to inherent limitations of standard statistical analyses against large, non-parametric community datasets. By applying community ecological analyses to evaluate the correlation between host and clinical factors and the chronic wound bacterial microbiota, it is possible to explore the associations between non-culturable bacteria with wound pathogenesis, chronicity, and infection.

In this study, we used 16S rRNA gene-based pyrosequencing analysis of the V3 region to characterize wound microbiota from a cohort of chronic wound patients and assessed the impact of diabetes and antibiotic therapy on bacterial communities. We have chosen the V3 region for its demonstrated ability to resolve bacterial taxa and produce comparable results to full-length (V1-V9) 16S rRNA gene sequences in 98.93% and 97.99% at the bacterial family and genus level, respectively, against human gut microbiota data [17]. We found that wounds were colonized by a wide-range of bacterial taxa including fastidious anaerobic pathogens that were not observed by culture-based analyses. Using community ecological analyses, we found evidence supporting clinical observations that diabetics were more likely to be colonized with Streptococcaceae and determined that recent antibiotic use was associated with increased Pseudomonadaceae colonization.

Results

Study Population

The demographic and clinical characteristics of the 24 participants included in the current analyses are listed in Table 1. Thirty-two individual wound samples were collected from the 24 participants at different times during the study. Fourteen of the wound samples were collected from patients who had been treated with topical or systemic antibiotics within 2 weeks prior to sample collection. The antibiotics administered prior to sample collection are listed in Table 2. Only 3 of the 14 antibiotic-treated wound samples were collected from participants who entered the study without receiving systemic or topical antibiotics but were treated during the period of observation.

Diverse Bacterial Communities Revealed by 16S rRNA gene-based Pyrosequencing Analyses

Bacterial taxonomic richness and diversity varied greatly among wounds examined in this study. Community richness and diversity were presented using Rarefaction and Shannon-Weaver Index plots, both of which provided insights into the structure and complexity of individual wound communities.

Table 1. Demographic and clinical characteristics of study participants.

Characteristics	Value
Age (SD)	57.2 (15.6)
Sex	
Male	N = 10 (41.6%)
Female	N = 14 (58.4%)
Race	
Black	N = 11 (46.0%)
Caucasian	N = 13 (54.0%)
Primary Diagnosis (Wound Type)	
Decubitus	N = 7
Neuropathic	N = 7
Venous Stasis	N = 3
Post-Surgical	N = 3
Other	N = 4
Diabetes Mellitus	N = 12 (50.0%)
Antibiotic (for wound samples, N = 32)	
Topical	
24 h	N = 5 (15.6%)
Systemic	
24 h	N = 5 (15.6%)
2 weeks	N = 10 (31.3%)
Any antibiotic use in past 2 weeks	N = 14 (43.8%)

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Rarefaction plots (Figure 1A) appear as two-component functions with a rapid increase in bacterial taxa observed until ~50 sequences are sampled; after which, a second component

Table 2. Systemic antibiotics used within two weeks of sample collection.

Wound	Antibiotic
TG03	Unknown*
WS06	Keflex
WS08	Levofloxacin 750
WS10	Doxycycline
WS18	Bactrim DS, Flagyl
WS19	Clindamycin
WS20	Bactrim, Clindamycin
WS26	Levofloxacin
WS27	Clindamycin
WS30	Vantin, Flagyl
WS31	Bactrim
WS32	Bactrim DS, Flagyl
WS36	Cipro
WS38	Bactrim DS, Clindamycin
WS39	Bactrim DS

*The patient reported antibiotic use within the previous two weeks, but did not know the name of the antibiotic.

doi:10.1371/journal.pone.0006462.t002

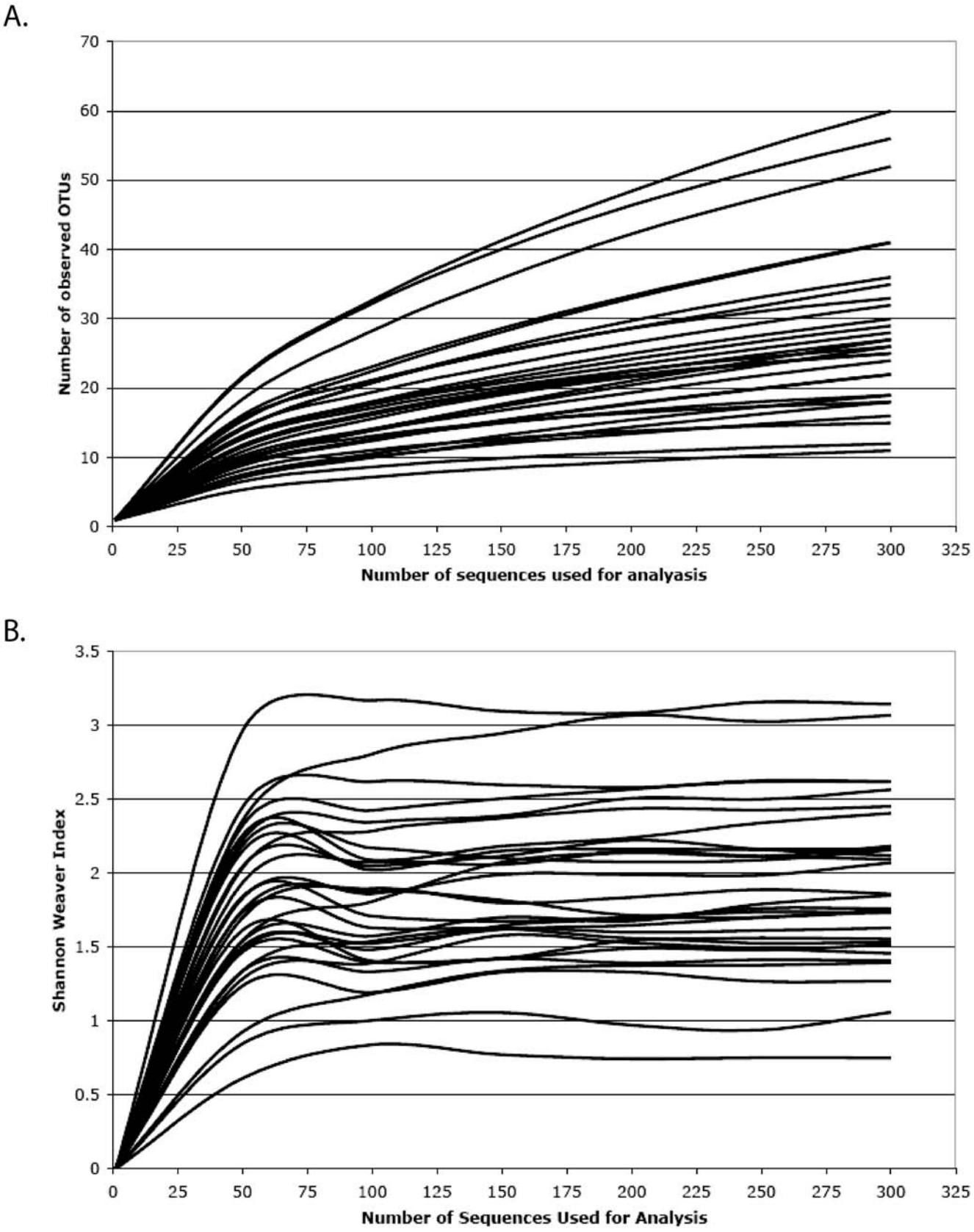

Figure 1. Rarefaction and Shannon Weaver index analyses were performed for each wound specimen. (A) Rarefaction curves were used to estimate richness (i.e., number of unique bacterial taxa) among samples. (B) Shannon Weaver Index curves were used estimate diversity (i.e., a combined assessment of the number of unique bacterial taxa and their abundance) among samples.
doi:10.1371/journal.pone.0006462.g001

with a lesser slope occurs in all cases. The first component includes the higher-frequency taxa that dominate the wound, while the second component represents less-frequent taxa. In individual wounds, the high-frequency taxa are as few as six or as great 25, with an average of ~10. Less-frequent taxa more than double the observed taxonomic richness but not until 250 to 300 sequences have been analyzed.

Diversity value plots (Figure 1B) are driven entirely by the high-frequency taxa. Similar to the rarefaction plots, the diversity values increase until ~50 sequences have been sampled. After which, the values stabilize with little change even when 300 sequences are sampled. This diversity index is based upon both the number of taxa and their frequency in the community; with numerous rare taxa having little effect on the final value. From these data, it is clear that 300 sequences are sufficient to estimate the bacterial community diversity values in individual wounds.

Bacterial community structure is determined through a mixture of high-frequency and low-frequency taxa, which are both potentially important to wound ecology and healing. Why some wounds have richer and more diverse communities is not apparent in these data and could be due to host, environmental or even stochastic processes. No trends in bacterial taxa richness or diversity values were evident among the diabetic or antimicrobial therapy groups (data not presented), though larger studies might be needed to detect these effects.

Taxonomic assignments were made with a bootstrap confidence range at ≥95% using the RDP Naïve Bayesian Classifier. The level of taxonomic resolution varied among sequence types identified in this study. While nearly all (98.8%) of the sequences analyzed were identified to the phylum level, the proportion of sequences successfully assigned to lower taxonomic groups decreased to 97.5% at the class level, 95.4% at the order level, 93.2% at the family level and then precipitously to 72.6% at the genus level. Importantly, only 53% of the Proteobacteria identified in this study were classified to the genus level.

Compared to culture-based analyses, 16S rRNA gene-based analyses revealed greater complexity at each taxonomic level (Table 3), identifying 44 bacterial families among the 32 wound samples (Figure 2). Most families were rare among samples and in low abundance when detected, thus confirming Shannon-Weaver Index analyses.

Only nine out of the 44 bacterial families identified by 16S rRNA gene-based analyses were also successfully cultured in this study (in red, Figure 2). Many of the 35 discordant families (those detected by 16S rRNA gene-based analyses, but not by culture-based analyses) were relatively rare among samples; however, one family—Clostridiales family XI—was both prevalent (present in 78% of the samples) and abundant (>10% of sequences on average in positive samples). Five genera belonging to this family were identified among the wound samples including: *Anaerococcus*, *Finegoldia*, *Helcococcus*, *Parvimonas*, and *Peptoniphilus*.

Percent agreement between 16S rRNA gene-based and culture-based analyses averaged 71% for bacterial families that were cultured at least once (Table 4). 16S rRNA gene-based analyses were consistently equal to or more sensitive than culture-based methods at detecting these nine bacterial families. Culture-positive/pyrosequencing-negative discordance was rare; thus, most of the disagreement was due to culture-negative/pyrosequencing-positive discordance.

Antibiotic Therapy and Wound Microbiota

We compared bacterial communities in wounds from patients recently treated with antibiotics to those from untreated patients using non-metric multidimensional scaling (nMDS) and multi-response permutation procedure (MRPP). We further identified the bacterial families that best distinguish the two antibiotic use groups using Dufrêne & Legendre indicator analysis.

The nMDS and MRPP analyses showed significantly different bacterial communities in patients that were untreated or treated with antibiotic in the two weeks prior to sample collection (Figure 3). The indicator analyses found that recent antibiotic use was associated with increased abundance of Corynebacteriaceae, Oxalobacteraceae, and Pseudomonadaceae.

We compared the prevalence of Corynebacteriaceae, Oxalobacteraceae, and Pseudomonadaceae between antibiotic use groups by calculating the mean difference in sequence counts and performing univariate pair-wise comparisons using a t-test statistic and Monte Carlo-based re-sampling. We found a large proportional increase in each of these indicator taxa in the antibiotic treated group, but the within-group variances were also large (Table 5). Univariate comparisons showed that Pseudomonadaceae was significantly higher in wounds from antibiotic treated participants compared to untreated participants. The increases in Corynebacteriaceae and Oxalobacteraceae were not significant after the Bonferroni correction (Table 5). To further evaluate the association between Pseudomonadaceae and antibiotic use, we examined the prevalence data in three participants from whom the pre- and post-antibiotic data were available. In this small subset, we found a significant increase in Pseudomonadaceae after antibiotic therapy (~27 fold average increase; $p = 0.047$).

Diabetes and Wound Microbiota

We further evaluated the association between diabetes and chronic wound microbiota using nMDS, MRPP, indicator analysis, and indicator prevalence as described above. The nMDS and MRPP analyses did not reveal a significant difference between the diabetic and non-diabetic wound microbiota on the community level (data not shown). Indicator analysis showed that differences in

Table 3. Estimated complexity at different taxonomic levels by pyrosequencing and culture.

	Pyrosequencing			Culture		
Taxonomiclevel	**Total # Among Samples**	**Range**	**Mean (SD)**	**Total # Among Samples**	**Range**	**Mean (SD)**
Phylum	6	2–5	3.3 (0.9)	4	1–4	1.9 (0.9)
Class	13	3–9	5.4 (1.4)	5	1–4	1.9 (0.9)
Order	23	3–13	7.8 (2.4)	7	1–5	2.5 (1.2)
Family	44	3–22	10.0 (3.9)	9	1–5	2.5 (1.2)
Genera	58	3–24	9.4 (4.6)	14	1–6	2.7 (1.5)

doi:10.1371/journal.pone.0006462.t003

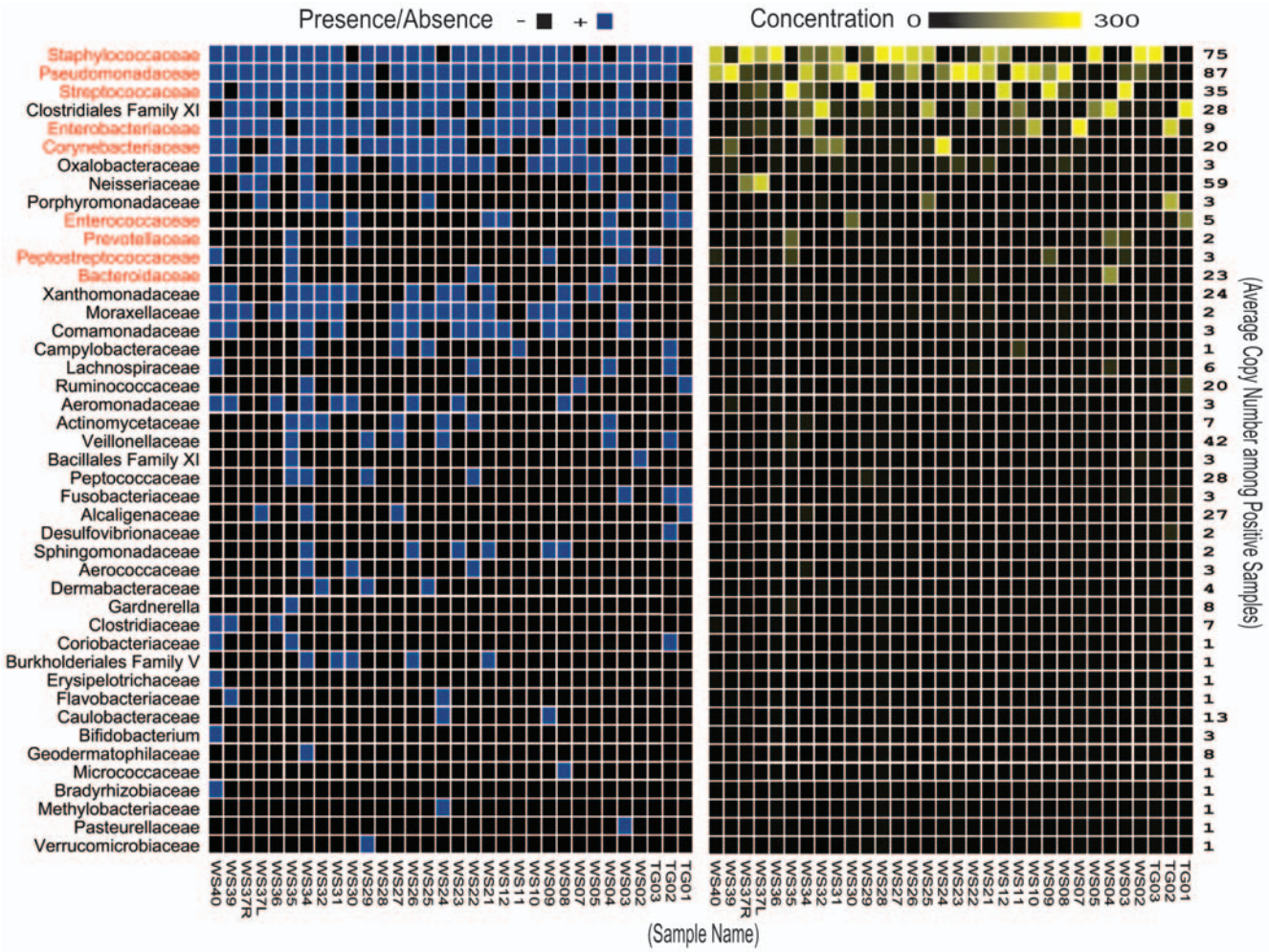

Figure 2. Heat map analysis of the 44 bacterial families detected using 16S rRNA gene-based pyrosequencing among chronic wound samples. The families in red are those that were successfully cultured at least once during the study. The presence/absence plot on the left shows the bacteria present in each of the wound samples. The abundance plot on the right shows the number of 16S rRNA gene pyrosequences (300 maximum) in each of the wound samples. The average copy number per positive sample for each detected bacterial family is shown on the far right. Many rare bacterial families are only visible on the presence/absence plot on the left.
doi:10.1371/journal.pone.0006462.g002

Streptococcaceae prevalence best distinguished the diabetic from non-diabetic wound microbiota. Prevalence comparisons of Streptococcaceae in diabetic wounds (Mean = 49.83, SD = 69.97) versus non-diabetic wounds (Mean = 3.92, SD = 11.54) showed large between-group and within-group variances. The difference in Streptococcaceae prevalence between diabetic and non-diabetic wounds was significant ($p = 0.015$) using the Monte Carlo method.

Interaction Between Diabetes And Antibiotic Use On The Wound Microbiota

At the genus level, indicator analysis showed that *Streptococcus* was a shared indicator in antibiotic use and diabetes, with a decreased prevalence of *Streptococcus* in antibiotic treated wounds compared to untreated wounds and an increased prevalence of *Streptococcus* in diabetic wounds compared to non-diabetic wounds (data not shown).

We assessed the presence of interaction between diabetes and antibiotic use on the community-scale using nMDS and MRPP, which revealed a significant interaction between diabetes and antibiotic use (data not shown).

We assessed interaction between diabetes and antibiotic use in *Streptococcus* colonization using multiple logistic regression. Antibiotic use was associated with a 41% reduction in risk of *Streptococcus* colonization in diabetics ($p = 0.009$) but no significant risk reduction in non-diabetics ($p = 0.21$). Additionally, in those not recently treated with antibiotics, the diabetic wounds were 63 times more likely to be colonized with *Streptococcus* than the non-diabetic wounds (OR = 63, 95% CI = 3.32, 1194).

Discussion

Modern molecular tools such as 16S rRNA gene-based pyrosequencing provide powerful means to define chronic wound bacteria. We found that chronic wounds supported complex microbial communities comprised of a wide-range of bacterial taxa including fastidious anaerobic bacteria that were not observed using culture-based methods. The bacterial wound communities characterized in this study were similar in composition to those reported by other groups using 16S rRNA gene-based methods [14,16]. The number and proportion of bacterial taxa ranged greatly in individual wounds. Additional research involving longitudinal sampling is needed to understand the dynamics of bacterial communities in chronic wounds.

Table 4. Comparison between pyrosequencing and culture for detecting the nine bacterial families that were successfully cultured at least once, among all wound samples (n = 32).

	Pyro (−)	Pyro (+)	Total	Percent Agreement
Bacteroidaceae				93.75
Culture (−)	28	1	29	
Culture (+)	1	2	3	
Total	29	3	32	
Corynebacteriaceae				50.00
Culture (−)	10	14	24	
Culture (+)	2	6	8	
Total	12	20	32	
Enterobacteriaceae				56.25
Culture (−)	7	13	20	
Culture (+)	1	11	12	
Total	8	24	32	
Enterococcaceae				87.50
Culture (−)	24	2	26	
Culture (+)	2	4	6	
Total	26	6	32	
Peptostreptococcaceae				78.13
Culture (−)	25	5	30	
Culture (+)	2	0	2	
Total	27	5	32	
Prevotellaceae				81.25
Culture (−)	25	3	28	
Culture (+)	3	1	4	
Total	28	4	32	
Pseudomonadaceae				31.25
Culture (−)	1	21	22	
Culture (+)	1	9	10	
Total	2	30	32	
Staphylococcaceae				75.00
Culture (−)	3	7	10	
Culture (+)	1	21	22	
Total	4	28	32	
Streptococcaceae				87.50
Culture (−)	16	4	20	
Culture (+)	0	12	12	
Total	16	16	32	

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Not surprisingly, bacterial diversity was substantially higher when determined by 16S rRNA gene-based pyrosequencing analysis as compared to the culture-based analyses. The limitations of culture-based methods to characterize diverse bacterial communities from environmental and clinical samples have been noted previously; however, many organisms missed by culture-based methods in the current study were theoretically culturable using conventional methods. Some of the organisms that were missed by culture-based methods were proportionally rare and may have been masked by more dominant organisms in the culture media. Other organisms, such as those belonging to the Neisseriaceae and Campylobacteriaceae families, are fastidious and thus require special culture media that are not typically used when culturing wounds in clinical laboratories. Obligate anaerobes, such as Clostridiales family XI, are particularly difficult to grow and were not identified using culture-based methods in the current study. Using 16S rRNA gene-based sequence analysis, we identified bacteria from Clostridiales family XI in 25 of the 32 wounds analyzed. Five genera from this family were identified: *Anaerococcus*, *Finegoldia*, *Helcococcus*, *Parvimonas*, and *Peptoniphilus*. Complex anaerobic microbiota that include the Clostridiales family XI have been associated with diseases such as bacterial vaginosis [18,19], diabetic foot ulcers [16,20], necrotizing fasciitis [21], and periodontal disease [22,23]. Thus, the data presented here highlight the limitations of routine clinical culture to detect potentially important fastidious pathogens.

We compared 16S rRNA gene-based pyrosequencing to culture-based analyses for detecting bacterial taxa that were cultured at least once during the study. In this analysis, we found that detection of these culturable bacteria was consistently greater by 16S rRNA gene-based pyrosequencing as compared to culture-based methods. While culture-negative/pyrosequencing-positive discordant pairs were common, culture-positive/pyrosequencing-negative discordant pairs were rare. All bacterial families identified by culture-based methods were targeted by the amplification primers used in the current study; therefore, insufficient sampling is the most likely explanation for the rare culture-positive/pyrosequencing-negative discordant pairs [24]. In contrast, there are several possible explanations for culture-negative/pyrosequencing-positive discordant pairs, including: 1) *Molecular detection of viable/non-culturable bacteria*. Viable/non-culturable bacteria may include non-planktonic bacteria existing in biofilms, which are common in chronic wounds; 2) *Molecular detection of bacteria that were proportionately rare within the community and masked by more dominant bacteria in culture media*. This is an expected limitation of using non-selective culture media; 3) *Molecular detection of DNA from dead (non-viable) bacteria*. Detecting DNA from dead bacteria is a common criticism of using DNA-based molecular methods to characterize microbial communities. While we acknowledge this potential bias, our data suggest that underestimation by culture-based methods is far more likely. Strategies to reduce or eliminate nucleic acids from dead bacteria, such as incorporation of DNA digestion steps prior to cell lysis, may help minimize detection of dead bacteria. Another approach would be to perform RNA (cDNA)-based analysis, which would identify bacteria that are metabolically active.

The V3 hypervariable region is one of the most phylogenetically informative regions of the 16S gene, but this study illustrates the limitations of this region for taxonomic assignment using the RDP classifier. Previous work with the RDP classifier indicated that 83.2% of the bacteria in the Bergey corpus could be accurately assigned to the appropriate genus using 200 base segments of the 16S gene. Three phyla—Firmicutes, Proteobacteria, and Actinobacteria—were the most commonly misclassified [25]. The 16S sequences used in the current study were all greater than or equal to 200 bases, with most (89.1%) of the sequences falling into Firmicutes, Proteobacteria, and Actinobacteria phyla. High percentages of the Firmicutes and Actinobacteria sequences were successfully assigned to the genus level, 88.0% and 82.3%, respectively; however, only 53.0% of the Proteobacteria sequences were successfully assigned to the genus level. Most of the Proteobacteria sequences belonged to four families: Pseudomonadaceae, Enterobacteriaceae, Oxalobacteraceae and Neisseriaceae. Varying proportions of all four families were successfully assigned to the genus level: 69.2%, 29.0%, 23.8% and 1.8%, respectively. A combination of sequence homoplasy and inaccurate database assignments (i.e., falsely assigned reference

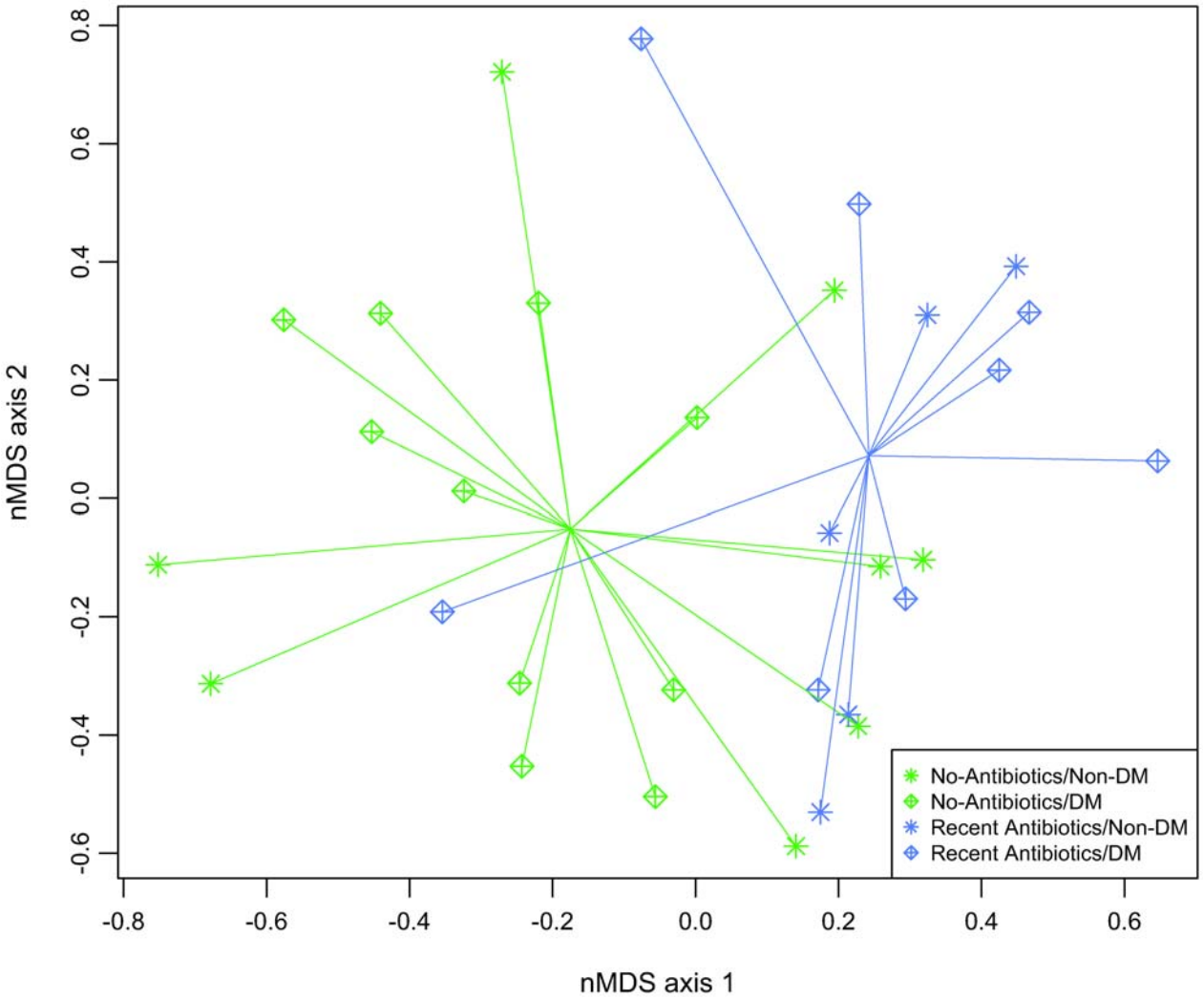

Figure 3. The nMDS ordination plot comparing wound bacterial communities from antibiotic treated participants and untreated participants. Each data point in nMDS plot represent the bacterial community identified from a single wound specimen. Comparison using MRPP found that the antibiotic treated and untreated wound microbiota are significantly different ($p = 0.0069$).
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sequences in the RDP database) could have contributed to our inability to assign Proteobacteria sequences to taxonomic groups below the family level.

Recent antibiotic use was associated with increased Pseudomonadaceae colonization in the current study. A similar association was reported previously in a study of tracheal colonization among critically ill, intubated patients [26]. In this earlier study, daily endotracheal aspirates were collected from patients after intubation. Bacterial communities from six of the seven patients shifted from relatively diverse communities to *Pseudomonas*-dominated

Table 5. Comparison of indicator species prevalence (out of n = 300 sequences for each sample) between untreated and antibiotic treated wounds.

	No Recent ABx	Recent ABx		
Taxonomic Group	**Mean (SD)**	**Mean (SD)**	**Δ Mean**	**Empirical p-value***
Corynebacteriaceae	2.5 (3.6)	25.4 (47.1)	22.9	0.019
Oxalobacteraceae	2.9 (6.3)	9.1 (8.8)	6.2	0.020
Pseudomonadaceae	39.9 (51.0)	110.1 (75.2)	70.2	0.0046**

*The empirical p-values comparing the prevalence of indicator species between the two antibiotic use groups were generated using the Monte Carlo method. The statistical significance level after the Bonferroni correction was 0.05/3 = 0.017.
**The increase in *Pseudomonadaceae* in the antibiotic treated group was significant at $p = 0.0046 < 0.017$.
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communities with the administration of antibiotics. Interestingly, *Pseudomonas* isolates collected from these patients were susceptible to the administered antibiotics in laboratory drug-susceptibility tests. It was hypothesized that this paradoxical finding was the result of differential susceptibility of *Pseudomonas* growing planktonically versus those growing in biofilms [26]. Biofilms are thought to be an important factor contributing to the chronicity of certain non-healing wounds. Administration of antibiotics may select for biofilm-producing organisms such as *Pseudomonas* and delay rather than aid wound healing.

One of the limitations of our study was its observational design, which may have resulted in selection bias. Participants were not excluded from the study based on prior therapies and eight patients entered the study having been treated with antibiotics within the previous two weeks. Those participants entering the study with recent exposure to antibiotics may have been treated in response to pre-existing *Pseudomonas* colonization or infection, which may have biased the observed association between antibiotic use and increased *Pseudomonas* colonization. Three participants entered the study without recent exposure to antibiotics and were treated during the study. Sub-analyses of these three participants revealed a significant increase in *Pseudomonas* abundance after antibiotic treatment. These data support the hypothesis that antibiotic use selected for increased *Pseudomonas* colonization, but additional prospective studies will have to be conducted to confirm these findings.

The 16S rRNA gene-based pyrosequencing analysis confirmed our clinical observations indicating that diabetics were significantly more likely to be colonized with *Streptococcus*. Increased *Streptococcus* colonization may be an important factor contributing to the disproportionate morbidity associated with chronic wounds among diabetics compared to non-diabetics [27]. Antibiotic use was associated with decreased *Streptococcus* colonization among diabetics and thus may be a suitable therapeutic option for treating diabetic patients with *Streptococcus* infections. Further studies are needed to confirm the association between diabetes and *Streptococcus* colonization and to elucidate the biological basis for this association.

Currently, wound management is largely empirical and based on principles of reducing bacterial load and preventing infection [28]; however, the complexity of the wound environment makes it likely that antimicrobial therapy could result in unintended consequences. We have little prospective data on the microbiological response to antimicrobial wound therapies. Thus, application of 16S rRNA gene-based pyrosequencing to characterize wound microbial communities with respect to clinical outcomes and therapeutic interventions (particularly antibiotic treatments) will provide critical insights into the roles of microbiota in wound healing and the impacts of wound therapies.

Materials and Methods

Clinical specimens

The study was approved by the Johns Hopkins Institutional Review Board. Chronic wound tissue samples were collected from 24 patients attending the Johns Hopkins Wound Center, a tertiary wound center in Baltimore, MD. After consent and local anesthesia, tissue was collected from the wound base with a 3 mm curette. Tissue samples were evaluated by qualitative aerobic and anaerobic culture in a CLIA-certified laboratory as follows: An unweighed portion of the sample was homogenized in a 15 ml conical tube using a sterile swab. One drop of the homogenate was plated on selective and non-selective media including sheep blood agar, MacConkey and chocolate agar plates and grown aerobically at 37°C for 24 hours. Columbia agar (CNA), CDC agar, and BBE/LKV were used for the recovery of anaerobic organisms. Plates for anaerobic assessment were incubated in the BD anaerobic gas pouch system at 35°C for 4–7 days.

DNA isolation from wound tissue samples

Genomic DNA was extracted from wound samples using a bead-beating and enzymatic lysis protocol, followed by purification using a QIAamp DNA Mini Kit (Qiagen, USA). Briefly, frozen wound samples (10 to 100 mg) were thawed on ice then suspended in 0.5 ml of TE50 (10 mM Tris-HCl +50 mM EDTA, pH 8.0) solution and allowed to soak on ice for 5 min before being vortexed. The suspension was transferred to a clean, sterile bead-beating tube (MP Biomedicals, USA) and kept on ice. A lytic enzyme cocktail was prepared at the time of extraction and added to each sample as follows: 50 µl Lysozyme (450 kU ml^{-1}), 6 µl Mutanolysin (25 kU ml^{-1}), 3 µl Lysostaphin (4 kU ml^{-1}) and 41 µl TE50 for a final volume of 100 µl per sample. Samples were digested by incubating at 37°C for 60 min in a dry heat block before centrifugation at 1200 rpm for 1 min. To each digested sample, 750 mg of sterile 0.1 mm diameter zirconia silica beads (BioSpec, Products Inc. USA) were added. Bead-beating was performed for 1 min at 2100 rpm using a BioSpec Mini-Bead Beater-96. Following bead disruption, the tubes were centrifuged at 1200 rpm for 1 min. A total of 200 µl of crude lysate was transferred to a new, sterile microcentrifuge tube. To each tube, 25 µl of Proteinase K (20 mg/ml (>600 mAU/ml)) and 200 µl of Qiagen buffer AL were added. Samples were mixed by pulse-vortexing for 15 sec and then incubated at 56°C for 10 min before being centrifuged at 1200 rpm for 1 min. For each 200 µl crude lysate, 20 µl of 3 M sodium acetate, pH 5.5 was added followed by 200 µl of molecular grade ethanol (96–99.5%). Vortexing was repeated for an additional 15 sec before being centrifuged at 1200 rpm for 1 min. From this point onward, purification was carried out using the QIAmp DNA Purification from Blood or Body Fluids (Vacuum Protocol) as per manufacturer's instructions. Purified genomic DNA was stored at −80°C until analysis.

Pyrosequencing library synthesis for parallel tagged sequencing on the 454® platform

The 16S rRNA gene was amplified in two replicate 50 µl reaction volumes. In each 50 µl reaction, 3 µl was added to 47 µl of PCR reaction mix containing 450 nM of each broad range forward (5′-CCTACGGGAGGCAGCAGT-3′) and reverse primer (5′-GGACTACCAGGGTATCTAATCCTGTT-3′) [29], 1X PCR buffer without $MgCl_2$ (Invitrogen), 3 mM $MgCl_2$, 0.2 mM dNTP mix, 0.02 U platinum *Taq* (Invitrogen) using the following touch-down PCR condition: 90 s at 95°C for initial denaturation, 30 s at 95°C for denaturation, 30 s at 64°C for annealing, 30 s at 72°C for extension with the annealing temperature decreasing by 0.3°C for each subsequent cycle for 34 cycles, followed by 5 min at 72°C for final extension. Subsequent purification, blunt-end repair, adapter ligation, amplicon quantification and pooling, restriction digestion, and pyrosequencing library generation were carried according to a previously published protocol [30]. The sample-specific, palindromic, self-hybridizing barcodes used in the tagging reactions were generated using a self-complementary 8-nt barcode and a rare restriction site according the same protocol.

Pyrosequencing using the 454® platform

The pooled tagged single-stranded pyrosequencing library underwent fusion PCR and pyrosequencing using a Roche 454

FLX Pyrosequencer (Roche Life Sciences, USA) according to the manufacturer instructions [31] at the Institute for Genome Sciences, Genomic Resource Center.

Sequence processing

Experimental sequences were processed using a custom PERL script, which performed the following: the script filtered the sequence files and retained only sequences that were 200-nt or longer. It then searched for a single barcode sequence in each FASTA sequence, binned each sequence accordingly, and scanned each binned sequence for the 16S forward primer sequence. The script then trimmed off the forward primer sequence and oriented the remaining sequence such that all sequences begin with the 5′ end according to standard sense strand conventions. As a result of our processing, sequences that were shorter than 200-nt or had multiple barcode or primer motifs were excluded from the analysis. We included only sequences with the forward primer motif to ensure that the highly informative V3 region was available for taxonomic assignment. The trimmed sequences from each barcode bin were aligned using the NAST alignment tool (http://greengenes.lbl.gov) [32]. After alignment, the number of sequences examined per wound sample was equilibrized to 300 sequences by sampling randomly without replacement to facilitate subsequent taxa abundance analyses. Samples with fewer than 300 sequences were excluded. The cutoff of $n = 300$ was established based on richness (rarefaction) and diversity (Shannon-Weaver Index) analyses using DOTUR [34], which indicated that samples were sufficiently sampled after ≥ 300 sequences.

Taxonomic assignment

Unaligned, sequences in the equilibrated dataset were given taxonomic assignments at a bootstrap confidence range of $\geq 95\%$ using the Ribosomal Database Project's Naïve Bayesian Classifier tool (RDP classifier) [25,35].

Rarefaction and diversity analyses

Distance matrices based on taxa abundance were generated with the Dnadist tool of PHYLIP 3.67 using the default settings [33]. Rarefaction and Shannon Weaver index estimations were determined by DOTUR [34] and plotted in Microsoft Excel (Microsoft Corp., USA).

Statistical analyses

All statistical analyses were performed using our equilibrated dataset ($n = 300$ sequences per sample). Community-scale multivariate analyses including non-metric multidimensional scaling (nMDS), multiresponse permutation procedure (MRPP), and the Dufrêne & Legendre indicator analyses were performed in R [36] using statistical packages vegan [37], ecodist [38], BiodiversityR [39], and labdsv [40]. The nMDS analysis is a nonparametric ordination-based method for reducing ecological community data complexity and identifying meaningful relationships amongst communities, while the MRPP analysis is another nonparametric method for testing the null hypothesis of no-difference between communities by comparing the experimental with the expected within-group difference through an iterative randomization process. The indicator species analysis further identifies the bacterial taxa that are significantly unique to each environment (e.g., clinical variables of interest). The nonparametric nature of these ecological analysis methods is highly suitable for human bacterial community data, which are frequently zero-rich, highly-skewed, and non-normal and remains non-normally distributed post-data transformation. Significance level for MRPP and the Dufrêne & Legendre indicator analyses were set at $\alpha = 0.05$.

Comparative analysis of mean indicator prevlaence between environments (e.g. diabetics versus non-diabetics) were also performed in R using custom codes. Briefly, using the taxa abundance-based distance matrices, a t-statistic was calculated and the underlying null distribution was estimated using Monte-Carlo based resampling ($n = 10{,}000$ permutations). A two-tailed empirical p-value was generated by comparing the unpermuted data with the estimated null distribution. Significance levels were set at $\alpha = 0.05$ with the appropriate Bonferroni correction (α/n), with $n =$ number of tests performed for a single environment.

Assessment for the interaction between antibiotic use and diabetes and the percent agreements in the comparison of 16S rRNA gene-based and culture-based results were performed using multivariate logistic regression and the kappa-statistic, respectively in STATA 9 (StataCorp, USA).

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Author Contributions

Conceived and designed the experiments: LBP CML JHM YMF DME JRB PK GL JZ. Performed the experiments: CML JHM YMF JRB RR JR. Analyzed the data: LBP CML JHM YMF DME MA JRB RR CK PK GL JZ. Contributed reagents/materials/analysis tools: LBP DME MA JR CK PK GL JZ. Wrote the paper: LBP CML PK JZ. Edited the manuscript: JHM YMF DME. Contributed text to the final manuscript: MA JRB RR JR CK. Performed statistical analysis: CK. Critically reviewed the manuscript: GL.

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APPENDIX C. Plans for the next quarter.

In the next quarter we will combine the pyrosequencing data and the PhyloChip data to characterize all the major phylotypes found the wounds, identify targets for qPCR assays and begin our work developing these assays. Additionally, we will prepare both the MIHS and TGen sites to begin the prospective burn wound cohort study.